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Using Novel-Fusion Proteins

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<p>The provision of the T cell costimulatory molecule B7 to tumor cells can be an effective means of triggering a tumor specific cytolytic T cells response. One way to provide B7 to tumor cells would be to couple an anti-tumor antibody either directly to B7 or to an antibody to the B7 counterreceptor on T cells, CD28. To this end, a fusion protein has been developed which incorporates a single chain antibody fragment (scFv) to c-erbB-2 (Her2/neu), an oncogene product overexpressed by 30-50% of breast carcinomas, and the extracellular domain of B7-2 (CD86). Experiments are currently underway to determine if the fusion protein can bind to c-erbB-2 and CD28, and also to measure its ability to activate T-cells. In addition, single chain antibody fragments are currently being selected, using a phage display scFv library, which are specific for the T cell costimulatory receptor molecule CD28. Anti-CD28 scFv with a range of affinities will be isolated and the role of affinity and differential binding to T cell costimulatory receptors will be determined to identify their importance in the biology of T cell activation.</p>							
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1. Introduction

1.1. Breast Cancer and Current Strategies for Therapy

Breast cancer is the leading cause of cancer death in women. Current treatments include; i) the surgical removal of tumors or compete mastectomies; ii) radiation treatment; and iii) chemotherapy using radioisotopes, immunotoxins, or other drugs. None of these treatments are completely efficacious, and all have significant side effects (1, 2). The development of new therapeutics with significantly increased cure rates and dramatically reduced side effects is essential for the acceptable treatment of this disease.

1.2. Costimulation in Antigen Specific Immune Responses

Contact of the T cell receptor with MHC-peptide complexes is necessary but not sufficient for T-cell activation (3). If T cells receive the primary MHC-peptide signal, but a second, costimulatory signal is not received, then the T cells may enter a state of long-term unresponsiveness termed anergy, leaving them ineffective at eliminating the target cells (4, 5). The most significant receptor/ligand pair that can provide the costimulatory signal is thought to be the B7 molecule on antigen presenting cells (APC) interacting with CD28 on T cells (6, 7). After T cell activation, another B7 receptor is expressed on T cells and is thought to contribute to down-regulation of T cell responses (8). This second B7 receptor, termed CTLA-4, is expressed on T cells approximately 48 hours after activation and binds B7 molecules with a twenty fold higher affinity than CD28 (9). Thus, CTLA-4 may compete with CD28 for B7 binding, resulting in diminished CD28 T cell signaling (8). Two B7 molecules have been cloned to date, B7.1 and B7.2, and a third is thought to exist.

Both B7 and CD28 are glycoprotein members of the Ig supergene family of cell surface molecules with each having a single IgV domain (reviewed in ref. (10)). CD28 is generally expressed as a homodimer whereas B7 is monomeric. Two different B7 molecules have been cloned to date and have been termed B7-1 (CD80) (11) and B7-2 (CD86) (12). B7-2 has been shown to be constitutively expressed, whereas B7-1 expression appears to be induced after B cell activation. The expression of B7 molecules is generally limited to APC, including macrophages, activated B cells, and dendritic cells. Human and murine CD28 are highly homologous at the amino acid level with 69% amino acid identity and, therefore, human B7 (hB7) can effectively signal murine CD28 (Dr. John Imboden, personal communication).

1.3. Costimulatory Signaling and Cancer Therapy

The fact that the human immune system does not mount an effective attack against tumor cells suggests that one or more factors required for an antigen specific immune response is not present. Since the expression of B7 is generally limited to professional APC, it has been hypothesized that tumor cells lack the ability to provide the costimulatory signal to T cells, and thus cannot serve as APC in T cell activation. Indeed, recent experiments support the concept that provision of either

B7-1 or B7-2 to tumor cells can result in their elimination in a cytotoxic T lymphocyte (CTL) dependent manner (13 - 19). Several investigators have transfected murine tumor cells with either B7 alone, or in combination with MHC molecules, and determined their effects on tumor cell growth *in vivo* (13 - 19). In general, tumor cells transfected with B7 alone were shown to specifically activate CD8⁺ T cells, resulting in rejection of tumor cells *in vivo*. In some cases, T cell activation also resulted in protection against subsequent challenge with unmodified tumor cells. Human tumor cell lines transfected with B7 were also able to stimulate *in vitro* T cell proliferation and cytotoxic T cell responses (20). These experiments suggest that treatment with B7 transfected tumor cells could induce protective immunity by active immunization as a treatment for human cancers. Whether the B7 transfected tumor cells function solely by directly stimulating an anti-tumor CTL response or trigger the involvement of other immune cells is still unclear. However, these results suggest that provision of T cell costimulatory molecules to tumor cells will lead to tumor rejection.

1.4. Antibodies to the Breast Carcinoma Marker c-erbB-2

Specific delivery of molecules to tumor cells can be accomplished using antibody based targeting. A number of strategies have been devised for antibody based cancer therapy, including coupling tumor specific antibodies to immunotoxins, radioisotopes, and molecules that are involved in triggering an immune response. The underlying requirement of such strategies is the development of an anti-tumor antibody. Tumor specific antibody development requires identification of molecules whose expression is generally limited to tumor cells, or that are at least overexpressed by tumors. One viable choice for tumor targeting of breast cancer is the oncogene product c-erbB-2, which has been demonstrated to be overexpressed in 30-50% of human breast carcinomas (21, 22).

Until recently, mouse IgG were primarily used for antibody based tumor targeting, resulting in a number of limitations. IgG are large (150kD) molecules which diffuse slowly into tumors (1 mm every 2 days) (23). The large size of IgG also results in slow clearance from the body and poor tumor:normal organ ratios (24). If the antibody carries a toxic agent, significant bystander damage may result. Recent advances in molecular biology have made it possible to produce (Fab')₂ and Fab in *E. coli*, as well as even smaller single chain Fv molecules (scFv, 25 kD). The scFv consist of the heavy and light chain variable regions (VH and VL) connected by a flexible peptide linker which retain the binding properties of the IgG from which they were derived (25). Smaller antibody molecules, particularly scFv, are cleared from the blood more rapidly than IgG, and thus provide significantly greater targeting specificity (26). scFv also penetrate tumors much better than IgG in preclinical models (27).

Another disadvantage of murine antibodies or antibody fragments is that they are likely to be immunogenic when administered therapeutically. Murine or chimeric IgG are clearly immunogenic when administered to humans and some of the immune response is directed against the variable regions (28). The smaller size

antibody fragments should be less immunogenic, but this still may be a problem when repeated doses are required for therapy. Thus therapeutic antibodies would ideally be of human origin. Unfortunately, production of human antibodies using hybridoma technology has proven extremely difficult, especially antibodies against human proteins such as tumor antigens (29).

All of the above limitations can be overcome by taking advantage of recent advances in biotechnology to produce human antibody fragments directly in bacteria without immunization (reviewed in 30, 31). Bacterial libraries containing billions of human antibody fragments are created, from which binding antibody fragments can be selected by antigen. Antibody fragments are entirely human in sequence, and thus less immunogenic than murine or humanized antibodies. In addition, affinity can be increased *in vitro* to < 1 nM (32).

The technology described above has been used to produce high affinity human scFv which bind to c-erbB-2. The initial isolate, C6.5, came from a non-immune human scFv phage antibody library (33), bound c-erbB-2 with a K_d of 1.6×10^{-8} M, and resulted in specific binding to c-erbB-2 expressing SK-OV-3 tumor xenografts in scid mice (34). At 24 hours, however, less than 1% of the injected dose (ID) was retained per gram (gm) of tumor (34). To increase retention, mutant phage antibody libraries were created by chain shuffling, or by mutating the antibody complementarity determining regions (CDRs). A C6.5 mutant (C6L1) with a 6 fold increased affinity for c-erbB-2 ($K_d = 2.5 \times 10^{-9}$ M) was created by chain shuffling (35). The 24 hour tumor retention of C6L1 was almost twice as great as for C6.5 (1.13% ID/gm vs. 0.67% ID/gm, $p < 0.048$) with significantly higher tumor to normal organ ratios. Mutation of the C6.5 VL CDR3 and VH CDR3 resulted in a C6.5 mutant (C6MH3-B1) with a 100 fold increased affinity ($K_d = 1.6 \times 10^{-10}$ M)(36). C6MH3-B1 represents the highest affinity tumor targeting antibody produced by any means and *in vivo* biodistribution studies of this molecule are pending. In the process of producing C6MH3-B1, the necessary techniques to rapidly and efficiently create, select and characterize higher affinity phage antibodies has also been developed.

1.5. Bispecific Antibodies and Fusion Proteins Combining Anti-c-erbB-2 and Costimulatory Molecules

The high affinity human anti-c-erbB-2 scFv provide a means for delivering B7 to breast cancer cells for *in vivo* T cell activation. A single polypeptide chain fusion protein can be constructed consisting of anti-c-erbB-2 scFv and B7. The fusion protein would be entirely human in sequence, would bind to c-erbB-2 expressing breast tumor cells with high affinity, and should provide the costimulatory molecule for T cell activation. An alternative approach would be to provide costimulation by replacing the B7 portion of the fusion protein with a scFv directed against CD28. This could be accomplished by creating a bispecific antibody fragment consisting of anti-c-erbB-2 scFv and an anti-CD28 scFv .

Bispecific antibodies have been produced which trigger the immune system, however none attempt to activate T-cells via a costimulatory signal. For example,

bispecific antibodies have been generated which are composed of anti-tumor antibodies coupled to anti-CD3 (37, 38). A limitation of using CD3, however, is that crosslinking of CD3 by the bispecific antibodies results in T cell activation regardless of MHC expression, immunogenic peptide display, or T cell restriction.

Thus there are theoretical advantages to activating T cells via costimulatory molecules compared to anti-CD3, or other conjugates. The three main advantages can be summed up as increased specificity, increased range of targets, and longevity of the therapeutic effect. Firstly, T cell activation via costimulatory molecules is antigen specific; the non-tumor killing associated with either CD3 crosslinking, general cytokine therapy, or immunotoxin or radioisotope conjugates would be prevented. For example, in anti-tumor therapy using an anti-c-erbB-2 antibody coupled to CD3, all cells expressing c-erbB-2 would be targeted for destruction, whereas only those cells displaying immunogenic peptides in the context of MHC molecules should be targeted when using anti-c-erbB-2/B7 or anti-CD28 chimeras. In addition, the severe toxicity associated with the use of immunotoxins or radioisotope antibody conjugates would be avoided. Secondly, tumor cells need not express c-erbB-2 to be targeted. Provided that immunogenic peptides displayed by MHC molecules are common between c-erbB-2 expressers and non-expressers, once a response is generated against a particular antigen, T cells should respond to that antigen regardless of the presence of the c-erbB-2 marker (16). The importance of this point should not be understated; even tumor cells which lose expression of the target marker will still be destroyed. A final advantage of activation via costimulatory molecules is the induction of protective immunity. Recurrences should not occur because the immune system will be primed for further responses to tumor growth.

1.6. Hypotheses

Hypothesis 1

Tumor antigen specific T cell activation can be achieved using a novel, bifunctional fusion protein which incorporates an anti-tumor antibody and the extracellular domain of the costimulatory molecule B7. The fusion protein should bind with high affinity to cancers and provide B7 for T cell costimulation, resulting in tumor specific T cell activation, proliferation, and tumor cell cytosis.

Hypothesis 2

Since the affinity of B7 for CD28 has been estimated at 4uM, a higher affinity interaction to signal T cells through CD28 may be more advantageous (39). Development of a number of anti-CD28 scFv with a wide range of affinities will be useful in exploring the relationship between affinity and T cell activation through the CD28 receptor. Anti-CD28 scFv with preferential binding to CD28 versus CTLA-4 may be more effective at T cell costimulation since CTLA-4 is thought to down-regulate T cell responses. Anti-CD28 scFv can be used with anti-c-erbB-2 scFv to make bispecific antibodies for tumor therapy.

2. Body

2.1 Experimental Methods

Construction of the scFv(c-erbB-2)-B7 gene

To generate the scFv(c-erbB-2)-B7 fusion proteins, the genes for CD80(B7-1) and CD86(B7-2) were PCR amplified from vectors containing the genes (gift of Dr. Lewis Lanier, DNAX) with primers containing *Apa*I or *Not*I restriction site overhangs and individually subcloned *Apa*I/*Not*I into pHenIX vectors. Anti-c-erbB-2 scFv genes were subsequently subcloned *Sfi*1/*Xho*1 into the pHenIX vectors already containing the B7 genes. Correct clones were identified by DNA sequencing.

Expression and purification of recombinant proteins

The scFv(c-erbB-2)-B7 constructs were subsequently subcloned *Sfi*1/*Not*1 from the pHenIX vectors into the mammalian expression vector pSecTag.B (Invitrogen) which contains the CMV promoter, Vk Ig leader sequence for protein secretion, and the *myc* epitope and (His)₆ tags for detection and purification, respectively. CHO cells were transfected by the calcium phosphate method and stable transfectants selected by growth in RPMI 1640 supplemented with 2mM glutamine, 10% FCS, and 1mg/ml G418. Fusion protein was expressed in CHO cells and purified by IMAC (40) exactly as described in (34).

Binding assays

The ability of scFv(c-erbB-2)-B7 to simultaneously bind both c-erbB-2 and to the B7 receptor was determined by enzyme linked immunosorbent assay (ELISA). Briefly, ELISA were performed with the extra cellular domain (ECD) of c-erbB-2 (gift of Jim Huston, Creative Biomolecules) immobilized on 96 well plates (Nunc; Maxisorp). Fusion protein was allowed to bind and detected by addition of CTLA-4Ig (gift of Dr. Mark de Boer), HRP conjugated anti-human Fc Ab, and incubation with peroxidase substrate followed by reading at 405nm by ELISA plate reader.

Preparation of phage and selection of phage antibody libraries

To rescue phagemid particles from the libraries, 10 ml of 2×TY-AMP-GLU were inoculated with an appropriate volume of bacteria (approximately 50 to 100 µl) from the library stocks to give an A₆₀₀ of 0.3 to 0.5 and grown for 30 min, shaking at 37°C. About 1×10^{12} plaque-forming units of VCS-M13 (Stratagene) particles were added and the mixture incubated at 37°C for 30 min without shaking followed by incubation at 37°C for 30 min with shaking. Cells were spun down, resuspended in 50 ml 2×TY broth containing 100 µg ampicillin/ml and 50 µg kanamycin/ml (2×TY-AMP-KAN), and grown overnight, shaking at 25°C. Phage particles were purified and concentrated by two PEG-precipitations (41), resuspended in 5 ml phosphate buffered saline (25 mM NaH₂PO₄, 125 mM NaCl, pH 7.0, PBS) and filtered through a 0.45 µ filter. The phage preparation consistently resulted in a titre of approximately 10¹³ transducing units (t.u.)/ml ampicillin-resistant clones.

The Sheets phage-antibody library (Dr. Michael Sheets et al., in press) was selected using 75 mm x 12 mm immunotubes (Nunc; Maxisorp) coated with either 1 ml CD28Ig(9) or human IgG1(Sigma) at varying concentrations (kindly provided by Dr. Peter Linsley) in phosphate buffered saline (PBS) overnight at 4°C. Tubes were blocked for 1 h at 37°C with 2% skimmed milk powder in PBS (2% MPBS) and the selection, washing, and elution were performed exactly as described (33) using phage at a concentration of 5.0×10^{12} t.u./ml with two noteworthy exceptions. Selection for CD28Ig binding phage may have occurred in the presence of competing quantities of human IgG1 and after elution from the CD28Ig coated immunotube, an additional incubation of phage in IgG1 coated immunotubes may have been performed, followed by elution. One third of the eluted phage was used to infect 10 ml log phase *E.coli* TG1, which were plated on TYE-AMP-GLU plates. The rescue-selection-plating cycle was repeated 4 times, after which clones were analyzed for binding by ELISA.

After each round of selection, phage were tested by polyclonal ELISA in microtiter plates coated with either CD28Ig or human IgG1. Binding was detected by use of an anti-M13 peroxidase conjugated antibody using ABTS as substrate.

Initial scFv characterization

Initial analysis for binding to CD28Ig was performed by ELISA using bacterial supernatant containing expressed scFv. Expression of scFv (42) was performed in 96 well microtitre plates exactly as described in (33). For ELISA, microtitre plates (Falcon 3912) were coated overnight at 4°C with 2 mg/ml of CD28Ig or IgG1 in PBS and then blocked with 2% MPBS for 1 hr at room temperature. Bacterial supernatants containing expressed scFv were added to wells and incubated at room temperature for 1.5 hours. Plates were washed six times (3x with TPBS and 3x with PBS) and binding of scFv detected via their C-terminal peptide tag (myc epitope tag (43) using anti-myc tag antibody (9E10, Santa Cruz Biotechnology) and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described (33). The number of unique binding scFv was determined by BstN1 fingerprinting and DNA sequencing.

2.2 Assumptions

In an effort to develop a therapeutic capable of activating an anti-tumor immune response, the construction of two different chimeric molecules was proposed. If each half of the chimeric molecules is available for binding, and the orientation of the molecules is appropriate for simultaneous binding to c-erbB-2 on tumor cells and to CD28 on T cells, then these molecules should be able to induce activation and proliferation of T cells. I have proposed two different ways of engaging CD28 (either via B7 or an anti-CD28 antibody) to increase the likelihood of producing a molecule with the proper orientation for binding.

Because this work takes advantage of techniques that are commonly performed in our laboratory, we anticipate that preparation of the fusion protein and bispecific antibody will be straightforward. The most time consuming part of the work will be isolation of an appropriate anti-CD28 scFv antibody fragment, which could take up to 6-18 months depending on the affinity desired. If the affinity of the initial isolates

are too low (less than 1.0×10^{-7} M), than *in vitro* affinity maturation will be performed as previously described (32). Once an anti-CD28 scFv is obtained, construction, expression, and purification of the bispecific antibody should be straightforward. The orientation of the two halves of the bispecific molecule should be adequate for the binding to their respective binding sites both individually on immobilized c-erbB-2 or B7, or simultaneously on adjacent cells based on similar bispecific antibody work (37).

2.3 Procedures

Generation and expression of scFv(c-erbB-2)-B7 fusion protein.

A chimeric fusion protein was engineered by genetic linkage of a scFv specific for the tumor antigen c-erbB-2 and the extra cellular domain of the T cell costimulatory molecule B7. The fusion protein genes were produced as described in the Experimental Methods section and subcloned into the pHenIX vector (Figure 1). The two fragments of the fusion protein genes are separated by a gene segment which encodes the 15 amino acid linker (G4S)3. This linker will join the C-terminus of the anti-c-erbB-2 scFv antibody fragment and the N-terminus of the B7 protein, allowing flexibility for binding of both proteins to their respective binding sites on adjacent cells. Initially, an attempt was made at expression in *E. Coli*, but solubility problems were encountered. Because the B7 molecule's extensive glycosylation may be important for solubility of the B7 protein, a mammalian expression system was investigated next. The scFv(c-erbB-2)-B7 fusion protein genes were subcloned into the mammalian expression vector pSecTag.B (Invitrogen) which contains the CMV promoter, Vk Ig leader sequence for protein secretion, and both a myc peptide tag for detection and a six histidine tag for purification by immobilized metal affinity chromatography (IMAC). The fusion protein gene was inserted into Chinese Hamster Ovary cells (CHO) and clones expressing the highest quantity of fusion protein isolated.

The production of six different fusion proteins was attempted. Three different anti-c-erbB-2 scFv of varying affinity were used (C6.5, ML3-9, C6MH3-B1 (B1)), as were both B7 genes (CD80 and CD86), resulting in six different combinations of anti-c-erbB-2 genes with B7 genes. However, only three of the six constructs were expressed at detectable levels in CHO cells, B1/CD86, ML3-9/CD80, and ML3-9/CD86.

Several hundred microgram quantities of each of the expressed scFv(c-erbB-2)-B7 fusion proteins were purified by IMAC and samples analyzed by SDS-PAGE and Western blotting (data not shown). The fusion proteins did not IMAC purify as single species as determined by SDS-PAGE and may need to undergo further purification on an anti-MYC tag Ab (9E10) column. Western analysis using the myc tag antibody, 9E10, showed a single, wide band around 90-95 kd. The size of this band may be due to the varying extent of glycosylation.

Binding experiments with the fusion protein.

To determine if the scFv(c-erbB-2)-B7 fusion proteins could simultaneously bind both c-erbB-2 and the B7 receptor, enzyme linked immunosorbent assays (ELISA) were developed. Microtiter plates were coated with the extra cellular

domain (ECD) of c-erbB-2 (gift of Jim Huston, Creative Biomolecules), blocked, and titrating quantities of fusion protein allowed to bind. One of the B7 receptors on T cells, CTLA-4 was used in this sandwich ELISA, because of its high affinity for B7. CTLA-4Ig, a chimeric, soluble fusion of CTLA-4(gift of Dr. Mark de Boer), was subsequently incubated in the plates. Simultaneous binding of the fusion proteins to both the c-erbB-2 ECD and to CTLA-4Ig could be detected by following with an peroxidase conjugated anti-human Fc antibody and peroxidase substrate.

Only one of the three fusion proteins produced, B1/CD86, gave a strong signal when tested by ELISA (Figure 2). Initial binding experiments using surface plasmon resonance (SPR), in a BIACore, have also shown that B1/CD86 is bifunctional (data not shown). When B1/CD86 is allowed to flow over a c-erbB-2 ECD coated surface, a resonance unit (RU) change is observed as the fusion protein binds the immobilized tumor antigen. Subsequent addition of CTLA-4Ig results in a further increase in the RU bound.

Anti-CD28 scFv antibody fragment generation.

Single chain antibody fragments (scFv) are currently being developed to the T cell costimulatory receptor molecule CD28 and the role of affinity and differential binding to T cell costimulatory receptors will be determined. Since the affinity of B7 for CD28 has been estimated at 4uM (39), a higher affinity interaction to signal T cells through CD28 may be more advantageous.

Several different anti-CD28 scFv have been isolated to date and are now being characterized. These scFv were isolated from a human phage antibody library by selecting on immobilized human CD28Ig (gift of Dr. Peter Linsley, Bristol Myers)(9). Because CD28Ig is a chimeric protein containing an IgG1 CH2-CH3 domain, two different strategies were utilized to minimize isolation of phage with specificity to the IgG1 portion of CD28Ig. Every round of selection was performed in the presence of soluble human IgG1 and nonspecific phage bound in solution were washed away before the elution step. In addition, after phage were eluted from immobilized CD28Ig in the last round of selection, the eluent was incubated for a half hour in a tube coated with human IgG1 and only the phage remaining in solution were used for infection.

To monitor the quality of each round of selection, polyclonal phage ELISA were performed on both CD28Ig and human IgG1 (Figure 3). A number of variables effect the selections, including antigen concentration, number of washes, and addition of secondary incubations with immobilized human IgG1. Many of the rounds of selection were repeated multiple times before proceeding to the next round.

Initial characterization of clones to identify antibody fragments which bind CD28.

To screen for individual clones that specifically bind to CD28Ig, mini-expressions of soluble scFv were performed in microtiter plates and tested by ELISA for binding to both CD28Ig and to human IgG1. Unlike the previously described polyclonal ELISA where large population of phage displaying scFv were tested, these ELISA test individual scFv clones for specificity. After overnight expressions of

scFv, supernatents containing the soluble scFv were transferred to 96 well plates coated with either CD28Ig or human IgG. Binding of soluble scFv was detected by incubation of wells with a murine IgG1 monoclonal antibody, 9E10, which recognizes the C-terminal myc peptide tag on the scFv, followed by a peroxidase conjugated anti-mouse antibody and substrate. A number of clones were tested positive for binding to both CD28Ig and human IgG1, however only a few clones were specific for CD28Ig (Table 1).

Clones which were tested as CD28 specific were characterized further by BstN1 fingerprinting of the DNA encoding the scFv antibody fragment. With few exceptions, each unique DNA fingerprint represents a unique scFv DNA sequence. The scFv DNA from at least two clones of each restriction pattern are being sequenced.

2.4 Results and Discussion

ScFv(anti-c-erbB-2)-B7 fusion protein.

The fusion protein incorporating the anti-c-erbB-2 scFv C6MH3-B1 and CD86 (B1/CD86) appears to be bifunctional by ELISA assays using c-erbB-2 coated plates and CTLA-4Ig. Since B1/CD86 appears to be able to function as envisioned in a CTLA-4Ig ELISA assay, it will be further tested in *in vitro* T cell assays for its ability to signal T cell responses through the CD28 receptor. Expression of the fusion protein in CHO cells has provided sufficient quantity of material for initial molecular characterization experiments and should be adequate for further studies. Should further purification of the fusion proteins may be necessary, secondary affinity purification can be performed using anti-MYC tag antibody, 9E10.

Presently, experiments are underway to show that B1/CD86 can provide the costimulatory signal to T cells. The ability of B1/CD86 to bind CD28 and costimulate a human T cell line, Jurkat, will be measured.

Anti-CD28 scFv

A phage-antibody library made from human antibody genes and human CD28Ig were used to generate anti-CD28Ig single chain antibody fragments. The use of human IgG1 aided in reducing the phage library of binders to the human CH2-CH3 domain of chimeric CD28Ig. In each round of selection, human IgG1 was used in solution as a competitor during incubations of phage on immobilized CD28Ig. In addition, during the fourth round, phage eluted from CD28Ig coated tubes were subsequently incubated on immobilized human IgG1. After four rounds of selection, 34/92 clones expressed scFv which bound to CD28Ig by ELISA whereas only 7/92 bound human IgG1. PCR fingerprinting of the positive clones with BstN1 showed that five unique restriction patterns exist. Therefore, after four rounds of selection utilizing human CD28Ig and human IgG1, five putative scFv were isolated which appear to be specific for the CD28 portion of chimeric CD28Ig.

Characterization of these clones will be performed by DNA sequence analysis, binding kinetics on CD28Ig, and T cell costimulation assays.

2.5 Recommendations to Statement of Work

Original Statement of Work (from proposal)

Specific Aim 1: Engineer a fusion protein incorporating anti-c-erbB-2 scFv and B7-2.

Task 1: Months 1-4: Genetically engineer fusion protein construct and confirm it is correct by DNA sequencing.

Task 2: Months 4-6 Express fusion protein and purify adequate quantities for in vitro evaluation.

Specific Aim 2: Engineer a bispecific antibody incorporating anti-c-erbB-2 and anti-CD28 scFvs.

Task 3: Months 1-6: Isolate scFv antibody fragments which bind CD28 by selecting a nonimmune scFv phage antibody library on immobilized CD28.

Task 4: Months 10-18: If necessary, increase the affinity of scFvs with the desired binding characteristics by creating mutant scFv phage antibody libraries and selecting on immobilized CD28.

Task 5: Months 21-24: Engineer bispecific antibody by genetic or chemical methods.

Specific Aim 3: Characterize and test the fusion protein and bispecific antibody.

Task 6: Months 4-36: Characterize anti-CD28 scFvs, fusion proteins, and bispecific antibodies with respect to DNA sequence, affinity, kinetics of binding, and T cell activation.

The project has been proceeding according to the timetable proposed with one minor exception. The start of work on producing anti-CD28 scFv was delayed for several months because CD28 material produced in our lab was not conducive to selections. Originally, CD28 protein generated in bacteria was to be used for selection of the scFv antibody. The structure/function of this CD28 protein was to be confirmed by analyzing the binding affinity and kinetics on B7-2, prior to performing selections. CD28 material produced in bacteria was found to be difficult to work with due to solubility problems. Therefore, an alternative source of CD28 was needed. Fortunately, Dr. Peter Linsley at Bristol Myer Squibb was kind enough to provide us with several milligrams of soluble, human CD28Ig (9).

3. Conclusions

1. A chimeric fusion protein has been generated that is specific both for the tumor antigen c-erbB-2 and for the T cell costimulatory molecule B7. Initial binding experiments demonstrate that the scFv(anti-c-erbB-2)-B7-1 is bifunctional in that it can bind to both c-erbB-2 and CTLA-4Ig simultaneously. Experiments are underway to test the ability of the fusion protein to costimulate T cells.
2. By using a human scFv phage library and selecting on CD28Ig, five putative single chain antibody fragments have been isolated. These anti-CD28 scFv are currently being characterized by DNA sequence analysis. Each different scFv will be tested for binding kinetics and T cell costimulatory ability.

4. References

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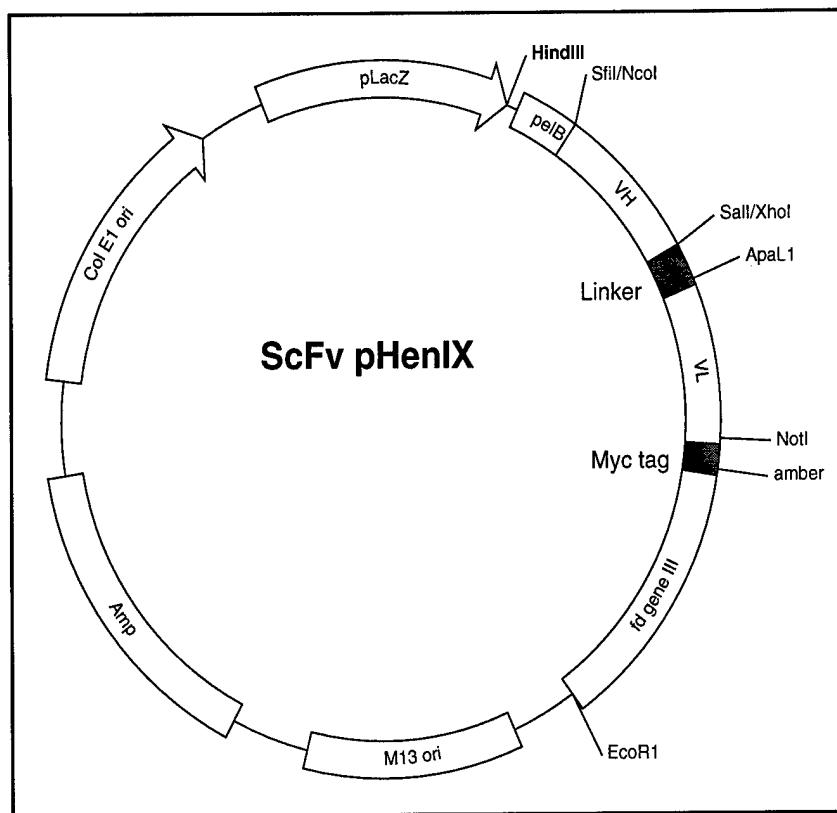


Figure 1. The expression vector scFv pHENIX.

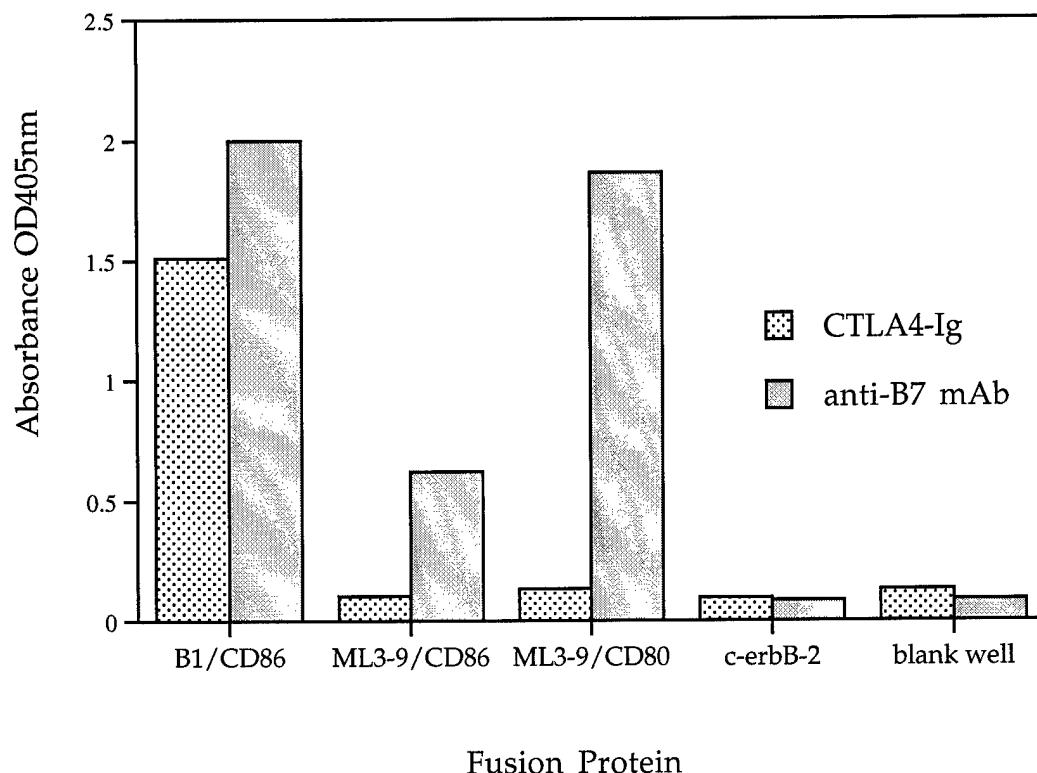


Figure 2. ELISA comparing ability of the fusion proteins to bind to c-erb-B2 ECD on the plate and to either anti-B7 mAb or CTLA-4Ig.

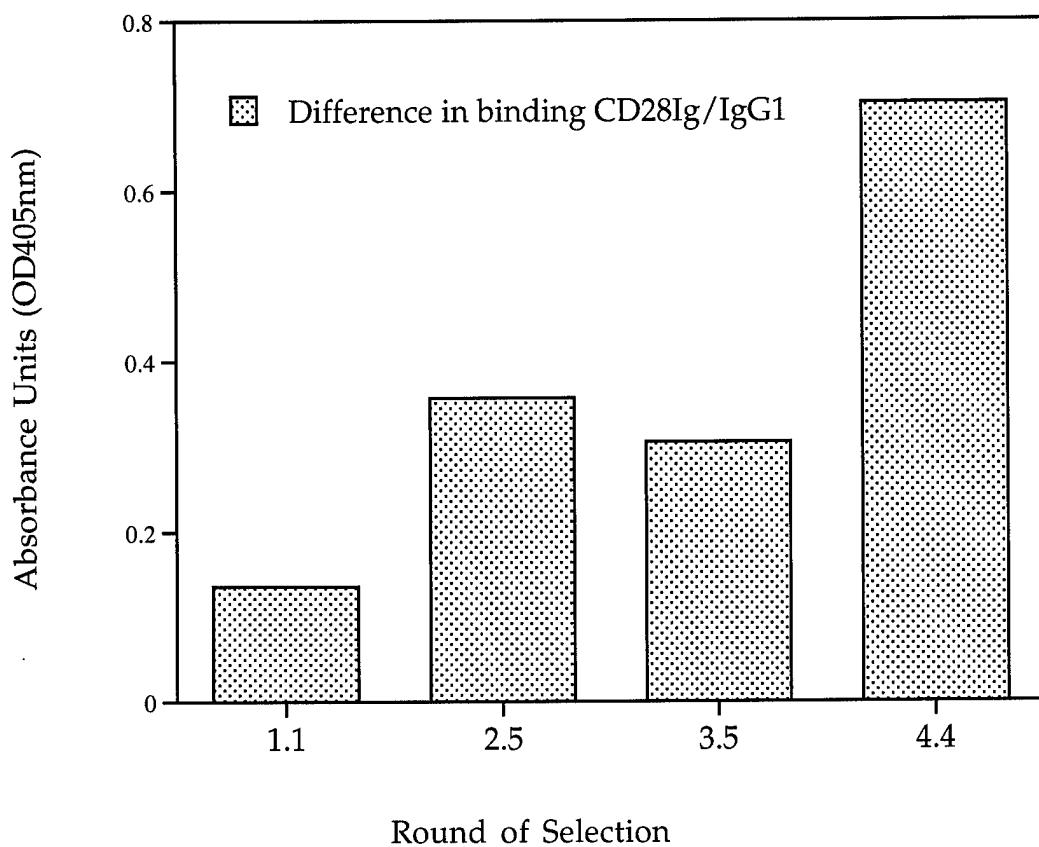


Figure 3. Polyclonal Phage ELISA - Values obtained by subtracting binding of polyclonal phage to human IgG1 from binding to CD28Ig.

TABLE 1. Selection conditions and resulting titer.

Round of selection	Selection conditions		
	[CD28Ig] ug/ml	[IgG1] ug/ml (solution)	Titer of Phage Eluted transducing units (t.u.)/ml
Round 1.1	10	0	1.3×10^6
Round 2.5	2	100	1×10^6
Round 3.5	2	40	2×10^9
Round 4.4	2	20	6×10^7